INTRODUCTION
1. INTRODUCTION

1.1. Complementary and Alternative Medicines (CAMs):
In the past few decades, Complementary and Alternative Medicinal Systems (CAMs) have received global acceptance. Botanical Dietary Supplements (BDS) have surfaced as a part of CAMs and more than 60 million people in the US alone are spending more than $600 million on these botanicals (Brevoort, 1994; Krochmal, 2004). They are perceived to be more close to nature and less harmful as compared to synthetic drugs which have various strong side effects. Indigenous medicinal systems that by and large were so far undocumented are moving towards global significance along with other CAMs like Chinese, Siddha & Unani, etc. The keen interest of the Pharma Industries in them and the increasing attention being given to these systems at the international level are evidence of the same (Berliner & Salmon, 1980).

World Health Organization datasheets indicate that more than 80% people resort to nonconventional medicinal practices, which include various philosophies, approaches and therapies which are not conventionally taught and/or do not exist in the formal curricula (WHO, 2002). These CAMs include highly heterogenous techniques ranging from diagnostics to therapeutics which include acupressure, acupuncture, aromatherapy, reflexology, herbal medicines etc.

Some frequently used synonyms for unconventional medicines are indigenous, unorthodox, alternative, folk, ethno, fringe, and unofficial medicine and/or healing. However, in the last decade or so, because of its growing demand and increasing use, these non-conventional medicines have acquired the new name of CAMs: Complementary and Alternative Medicines, which also shows an increasing acceptance of the same, by the medical fraternity.

The entry of CAMs into the mainstream healthcare system is becoming evident as an increasing number of pharmaceutical industries are showing keen interest by flooding the markets with more and more natural drugs most of which are herbal in nature (Ruggie, 2005).

1.1.1. CAM: Definitions:
The World Health Organization (WHO) has defined traditional medicine as “the sum total of all the knowledge and practices, whether explicable or not, used in diagnosis, prevention and
elimination of physical, mental or social imbalance and relying exclusively on practical experience and observation handed down from generation to generation, whether verbally or in writing” (WHO, 1978).

According to the definition used by the Cochrane Collaboration, ‘Complementary and Alternative Medicine’ is a broad domain of healing resources that encompasses all health systems, modalities, practices and their accompanying theories and beliefs, other than those intrinsic to the politically dominant health system of a particular society or culture in a given historical period. CAM includes all such practices and ideas defined by their users as preventing or treating illness or promoting health and well-being (Zollman & Vickers, 1999).

According to Eskinazi, alternative medicines can be defined as a broad set of health-care practices, which have pre-existed in the public domain and are not readily integrated into the dominant health care model, because of the challenges they pose to various cultural, scientific, medical and educational beliefs at large.

Complementary and alternative medicine is defined as “diagnosis, treatment and/or prevention which complements mainstream medicine by contributing to a common whole, by satisfying a demand not met by orthodoxy or by diversifying the conceptual frameworks of medicine” (Ernst et al., 1995).

However, the acronym CAMs, many a times obscures important distinctions between two concepts: ‘complementary’ and ‘alternative’. There are therapies that are complementary to medicines, which help in getting better results of the conventional medicines. And on the other hand, there are therapies used as alternatives or substitutes for medical treatments. Alternative therapies that replace conventional medical care, and are promoted as such, present specific risks and problems, which vary according to the health context of the user (Sanderson et al., 2006).

The term ‘CAMs’ is fast being replaced by ‘IMs’ (Integrative medicines), which is a new discipline of medicines and offers the best of the both worlds to the consumer as it rightly blends in both Allopathy and naturopathy (Willms & St Pierre- Hanson, 2008). This further makes it mandatory for each of these to be validated and there is a need for such validations for both allopathic
medicines for the side effects they show in specific individuals and for unconventional drugs for their efficacy *de facto*.

The above definitions focus on the factors that may play a significant role in the acceptance or rejection of such alternative therapies, be it acupressure and acupuncture or herbal drug therapy. With the emphasis on the knowledge base today, there has to be a scientific justification for each such alternative practice for its demand. Hence, the acceptance of these CAMs or IMs, now for the most part depends on the scientific proofs generated in support of the claims corresponding with existing remedies.

1.1.2. **Classification of CAMs:**
The National Centre for Complementary and Alternative Medicine (NCCAM) defines complementary and alternative medicine (CAM) practices as those not, at present, considered an integral part of conventional medicine.

NCCAM has grouped about 100 such CAM based therapies into five main domains, with some overlap across categories (Ruggie, 2005). The following table classifies various CAMs:

**Table.1.1. Classification of Complementary and Alternative Medicines (CAMs):**

<table>
<thead>
<tr>
<th>Categories of CAMs</th>
<th>Alternative Medical systems</th>
<th>Mind body interventions</th>
<th>Biological Therapies</th>
<th>Body based techniques</th>
<th>Energy Therapies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alternative Medical systems</td>
<td>Homeopathic medicines, Naturopathy, Traditional oriental Medicines, <em>Ayurveda, Siddha, Unani, Chinese</em></td>
<td>Cognitive behavioural therapy, Meditations, Biofeedback, Prayers, Mental healing, Music therapy, Dance therapy, Touch Therapy</td>
<td>BDS, Herbal medicines, Natural Dietary supplements, Special diets</td>
<td>Chiro practice, Acupressure, Acupuncture, Massage</td>
<td>Bio field therapies, Reiki, Qi Gong, Bioelectromagnetic therapies, Faith Healing</td>
</tr>
</tbody>
</table>

Source: http://nccam.nih.gov
1.13. CAMs and Medicinal plants:

Often, when we talk of unconventional medicines, we think of BDS- Botanical Dietary Supplements or Herbal medicines. According to Barnse et al. (2007) usage of natural product is the third most common CAMs self practiced by a majority of the US population. It also true for other continents to a greater extent.

Till the recent past, all the traditional practices, either in the form of ethnomedicinal wisdoms or religious or regional compilations of such practices like Ayurveda, Chinese medicines, Siddha and Unani have offered formulations to address various health ailments. However, with the increasing participation and interest of pharmaceutical industries, what began with a crude plant parts, today calls for the purification of an active principle.

The potential of CAMs to expand further either as a single new chemical entity or as a complex multi component botanical drug is great. One of the first drugs which was identified from the plant origin was Reserpine, from the herb Rauwolfia serpentina which is now known to cure high blood pressure. The plant was described many centuries ago in the Indian Ayurvedic monographs. There are many others, which were not known as medicines themselves, which include Quinine extracted from Cinchona bark, Digitonin extracted from Digitalis or Foxglove leaf, Morphine from Poppy seeds/herb and Vincristine from Rosy periwinkle (Clark, 1996; Tyler et al., 1988; Schultz et al., 2001).

In the recent past, around 20-30 such active principles have been isolated from various plant species, depending on their reported uses under CAMs. In the past decade or so, attention has been laid on the usage of medicinal plants to cure various health ailments as they are believed to be free of the negative side effects that synthetic or allopathic drugs have (Gijtenbeek et al., 1999; Johnson & William, 2002).

Thus, several plants, either having their mention in one of the alternative medicine compilations like Ayurveda, Siddha & Unani and Chinese medicinal systems or taken from the traditional wisdom of tribals or folklore have been established to be possessing important medicinal activities. Most of these systems offer a multicomponent remedy to cure an ailment and thus in most such cases there is a synergistic effect of various phytochemicals present in a plant.
1.1.4. CAMs: Need for research:

However, for most of these Botanical Dietary Supplements (BDS) or herbal medicines, which are claimed to be individualised, holistic etc., there is a need of a research based validation which can prove the effectiveness of these CAMs as equal or superior to allopathic medicines in curing the given ailment.

Compilations like Ayurveda, Siddha, Unani and Chinese medicines are well known to the world at large. Many of these multi component formulations have been consumed by people across the globe to self treat major and minor ailments and the efficacy of these have never been questioned. However, the World Health Organization and our national regulatory body Indian Council of Medical Research have now set various standards for the usage and validations of such plant materials. According to such reports, when an extract of a plant or a compound isolated from the plant and any compound formulation having plants, metals, minerals and animal products as ingredients has to be clinically evaluated for a therapeutic effect if it is not originally described in the texts of traditional systems, there has to be a specific validation method.

If, on the other hand, the method of preparation is different, it has to be treated as a new substance or new chemical entity (NCE) and the same type of acute, sub acute and chronic toxicity data will have to be generated as required by the regulatory authority for synthetic products before it is cleared for clinical evaluation.

On the other hand, an extract or a compound isolated from a plant and any compound formulation having plants, metals, minerals and animal products as ingredients which has never been in use before and has not ever been mentioned in ancient literature, should be treated as a new drug, and therefore, should undergo all regulatory requirements before being evaluated clinically (ICMR Reports, 2006).

According to Hyde there are certain problems in accepting CAMs in the existing state. It is still not a fool proof method to cure any disease and it grossly remains unclear whether all the claims made under various scriptures and folklores really hold any truth in them. There needs to be a proper categorization and prioritization and hence the necessity for evaluation, research and application.
While for any drug to be successful, clinical research data is nowadays considered to be the most significant research, preclinical processes of validation of efficacy can also provide a greater insight in the claims made as there are certain compounds that already have been reported to possess specific medicinal properties. By demonstrating significant concentrations of those in the plant part, it can safely be established that the same plant would also exhibit similar medicinal property. Thus, establishing the phytochemical markers becomes one of the mandatory steps to test any plant for the medicinal claims made about the same.

There are clear scientific indications that herbal CAMs will prove effective in preventing and treating many chronic diseases which remain an enigma for the modern world of medicines. It will also help in cost cutting in the health care domain. However, there are rigorous scientific verifications required before the CAM becomes a holistic medicinal cure (Engel & Straus, 2002).

For any conventional drug discovery process, the process begins with a basic research of preclinical studies which goes to the three phased clinical studies. Once the clinical studies show significant efficacy of the drug, the drug is approved by the Public Agency, which then goes to the market. The following Flow chart gives an overall picture of the drug discovery process.

**Fig. 1.1. Drug discovery process:**

![Drug discovery process diagram](source: NCCAM website)

The National Centre for Complementary and Alternative Medicines (NCCAMs) has however, taken a reverse approach to the CAM identification. Presuming its safety since it has been popular over generations, they have suggested a direct clinical trial of the Single Chemical Entities or a polyherbal formulation.
However, there are certain limitations to the approach taken up by the NCCAM. For most of the drug discovery processes, the drugs are synthesized in the laboratories. Thus, their purity, quality and quantity can be maneuvered to a greater extent. On the other hand, when we talk of the CAMs or BDS, the quality of the plant material depends on the surroundings and thus, different plant samples show different yields, which thus limits the study, especially when a crude herbal formulation is being considered for the same.

There are several reports that mention about intentional or accidental adulteration of plant material, variations in the yield of important phytochemicals and thus variation in the results obtained using those formulations (FDA paper, 2006).

In such circumstances, a preclinical analysis to standardize the yield and validate the presence of important medicinal compounds become a prerequisite and though, the conventional drug discovery procedure can be altered, the clinical trials cannot be directly adopted presuming the safety and quality of the plant material.

For many botanical products, the provisions for variations in clinical outcomes have to be made as the variations may occur with different cultivars, species and plant parts, like roots, shoots, whole plants, leaves, as well as growth and harvest conditions, maturity of the plant, seasonal variations etc. The variations can also be introduced by the way the product is derived or extracted (for example, alcoholic, tea, pressed juice), and the form in which it is provided. It is also important to verify that there are no adulterants and contaminants (such as proprietary drugs, insecticides, herbicides) in the final product. Only after establishing these, the clinical trials can be conducted.

Thus, there is a growing need for such validation processes which don't only scrutinize the properties claimed by various CAM systems, but also ensure a constant yield from the biological materials. For this, state of the art scientific tools must be applied to the empirical science of natural medications.
Fig. 1.2: Regulatory approaches for a botanical product

Source: Manufacturing and Control of Natural Drug, Investigation of Raw drug, NDA.
1.2. CAMs in India:
A Complementary and Alternative medicinal system is a new garb that the age old medicinal systems have donned. It is more like an umbrella term used for various chiropractices that have been existing in various regions across the globe.

In India, there are several generation old medicinal practices which have thrived and survived. Some are compiled and codified in the form of scriptures like Ayurveda, which is considered to be one of the most exhaustive CAMs compilation and others which are non codified, undocumented versions like ethnic folklores, which are becoming rarer with deforestation and urbanization of tribal youth (D'Cruz, 2002).

1.2.1. Ancient medicinal systems and their emergence in India:
In India, either in the form of various scriptures like Ayurveda, Siddha and Unani, or as various folklore practices developed and practiced by various tribal communities, CAMs have been in practice since the times civilizations came into existence.

Ayurveda, the oldest medical system in the Indian subcontinent, has alone reported approximately 2000 medicinal plant species, followed by the Siddha and Unani medical systems. The Charak Samhita, a classic written document on herbal therapy, reports the production of 340 herbal drugs for curing various diseases.

Currently, approximately 25% of drugs are derived from plants, and many others are synthetic analogues built on prototype compounds isolated from plant species in modern pharmacopoeia.

On one hand, our scriptures have detailed procedures to prepare various formulations using different plants to cure health problems, and on the other hand, the tribal traditions that make their ethnomedicinal wisdom, are secretive and exist on the fringe as the practitioners have several beliefs and taboos and do not easily share the knowledge with others (D'Cruz, 2002). And these ethno medicinal practices are the ones which have not yet got significant attention and thus there are many plant species which have not been properly worked on and verified.

WHO has listed 20,000 medicinal plants used in different parts of the world. Other estimates indicate the number to range between 35,000 and 73,000 worldwide (Lewington, 1993; Bhattarai & Karki, 2004). Some of the earliest uses of plants became widespread and with the passage of
time got organized and codified. Traditional medicinal systems employ relatively few species, viz. 500-600 in traditional Chinese medicine, 1100 in Tibetan medicine (Sowangpa), 1500 in Ayurveda, 450 in Homoeopathy, 342 in Unani, and 328 in Siddha systems. There are overlaps amongst various medicinal systems ranging from the part used to the process of formulation etc. However, there are certain plants which are exclusively found reported under a specific medicinal system (Shankar & Unnikrishnan, 2004).

Around 50,000 to 70,000 plant species are known to be used in traditional and modern medicinal systems throughout the world (Schippmann et al., 2006). About 3,000 MAP (Medicinal and Aromatic Plant) species are traded internationally (Lange & Schippmann, 1997), while an even larger number of MAP species are found in local, national, and regional trade.

There is a major proportion of plant species which has remained endemic to certain regions and thus to certain groups of people and which have equal or higher medicinal properties.

India is rich in both these resources. The codified versions like Ayurveda, Siddha systems of medicines have their origins in India. And the same India is home to several ethnic tribes which have their own non codified ethno medicinal wisdom, which is another pool of information waiting to be unleashed to the world of the medicinal sciences.

Due to lack of communication, intermingling and breeding of ideas, and varying ways of life, many of these earlier remedies survived only by word of mouth from generation to generation. This category of information and their uses still dominate the healing tradition in the world. Also, growing urbanization has played a role in keeping this information undocumented as tribal youth, in search of livelihood move to the cities and the interest in inheriting the wisdom decreases with the passage of time.

Thus, there is a dire need to document these fast disappearing ethnomedicinal usages and to scrutinize them to bring newer drugs to the market.

1.3. Medicinal plants in India:
Because of a variety of climatic conditions that prevail across India, the country is home to the third highest number of plant species in the world. The plant species that grow in the Western Ghats are completely different from those that are found in the Himalayan region. Probably, this
has given India an edge over other alternative herbal medicinal systems and the documentations that originate from India are much more exhaustive as compared to others.

1.3.1. India: Mega Biodiversity Hotspot

INDIA is the third biggest of the 12-mega biodiversity centres having about 8% of the world’s biodiversity wealth, which is distributed across 16 agro-climatic zones. In total, India is the home of around 47,000 different plant species having concentrated hotspots in the eastern Himalayas, Western Ghats and Andaman & Nicobar islands. Due to a variety of climatic conditions in its regions, the number of species specific to a particular climate are very high, thus resulting in a higher ranking as a biodiversity hot spot. Out of 17,000 species of higher plants reported to occur within India, 7500 are known to have medicinal uses (Shiva, 1996). However, officially documented plant species having medicinal potential are around 3000 only. This proportion of medicinal plants is the highest known in any other country against the existing flora of that country (Shiva, 1996; Kala et al., 2006).

Table. 1.2.: Medicinal Plants enlisted across various systems of Medicine

<table>
<thead>
<tr>
<th>Medicinal Systems</th>
<th>No. of plants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ayurveda</td>
<td>1549</td>
</tr>
<tr>
<td>Folk</td>
<td>4786</td>
</tr>
<tr>
<td>Homeo</td>
<td>492</td>
</tr>
<tr>
<td>Modern</td>
<td>193</td>
</tr>
<tr>
<td>Siddha</td>
<td>1151</td>
</tr>
<tr>
<td>Tibetan</td>
<td>250</td>
</tr>
<tr>
<td>Unani</td>
<td>493</td>
</tr>
</tbody>
</table>


Thus, India is the largest producer of medicinal plants and is rightly called the botanical garden of the world. There are currently about 250,000 registered medical practitioners of the Ayurvedic system which is more than 85% of the traditional systems practiced across the globe as compared to about 700,000 of the modern medicine system. In rural India, 70 per cent of the population is dependent on the traditional system of medicine, the Ayurveda (Bent & Ko, 2004; Dubey et al., 2004; Ahmedullah & Nayar, 1999; Sheth & Sharma, 2004).
1.4. Present concerns to medicinal plant ecosystems

Every time we talk of environmental management and resource conservations, we are reminded of the unabated loss of biodiversity occurring throughout the world. It is predicted that about half of the estimated 13.6 million species on earth may become extinct by the year 2050, unless we take appropriate measures to save them. The manifestations of the current biodiversity crisis also include the disappearance of many populations of species, depletion of genetic diversity, fragmentation, degradation or destruction of several unique habitats and ecosystems (Myers, 1987; Wilson, 1992; Myers, 1999; Pushpangadan & Nair, 2001). In the past decade and a half, continuous over exploitation of several plain species from the wild has resulted in the population decline of many high value medicinal plant species. (Indian Planning Commission Reports, 2000; Kala, 2003).

The potential causes for this undesired extinction of medicinal plant species are habitat specificity, narrow range of distribution, land-use disturbances, introduction of non-natives, habitat alteration, climatic changes, heavy livestock grazing, explosion of human population, fragmentation and degradation of population, population bottleneck and genetic drift (Kala, 2000; Weekley & Race, 2001; Oostermeijer et al., 2003).

There are certain local issues like groundwater depletion, meso-level questions like increasing water and air pollution, national problems like deforestation and non availability of cultivable land and global challenges like green house effect- which all at some extent have played a role in bringing the ecoequilibrium to this imbalance (Shah, 2002). However, unscrupulous and irresponsible encashment of medicinal plants has wreaked havoc on our ecology (Pant & Khanduri, 1998) resulting in catastrophes worldwide in the form of frequent natural calamities like tsunamis, earthquakes, floods and drought, sudden drops and rise in temperatures continuing the vicious cycle of depletion of the green canopy.

The prominent mode of obtaining medicinal plants is wild harvesting. Even till date, most of the industrial requirements are met through this mode (Lange, 1998). Though many medicinal plants are commonly available in the wild and can be freely harvested, uncontrolled collection and sale of large quantities of plant material from the forest has led to destruction of many forest plants especially the endemic species that have a restricted geographical distribution. Due to the growing economic importance and rampant harvesting of some such medicinally important species, they
have made it to the IUCN lists of endangered plants as critically endangered (Prasad and Patnaik, 1998). It is a matter of grave concern that such activities are still given a free hand and there is neither a domestic cultivation approach taken nor are any regulatory measures inflicted.

With climates becoming drastic due to green house effect and other environmental factors, it has become all the more difficult to grow these plants, many of which are season specific.

It has been estimated that nearly one third of India's plant species are endemics or near endemics, that is to say that these are not found outside India or they have only marginal presence outside our country. The World Conservation Union (IUCN) has published a compilation of threatened plants of the world in 1997 (Walter & Gillett, 1958) which enlists more than 34,000 vascular plant species in the threatened category, suggesting that across the globe, nearly 12.5 percent of known flowering plants are threatened with extinction. In the absence of any systematic assessment of such threatened medicinal plant species of India, it may be reasonable to extend the same proportion (12.5 percent) to the 6200 medicinal plant species enlisted in India. This suggests that about 744 medicinal plant species of India may be threatened with extinction and more than 300 of these are likely to be endemics or near endemics.

To rapidly assess and identify such threatened medicinal plant species and develop a focused conservation strategy is one of the major challenges conservationists in India have been facing. Since around 90 percent of India's flowering plant diversity is estimated to exist in our forests, the action for conservation of our medicinal plant resources has to take place in forest habitats and this will also need the full support of local communities who reside in and around the forests. Conservation programmes should be designed to simultaneously support projects for the revitalization of indigenous health cultures because biodiversity and cultural diversity have a symbiotic relationship and the loss of one will eventually lead to the loss of the other.

In such conditions, there has to be a planned scientific intervention which can help us re-establish the ecoequilibrium in situ. Many environmentalists and conservationists across the world have been strategically working on these issues and in the process have devised certain policies which can be of some help (Sundriyal & Sharma, 1995).
1.4.1. Scientific understanding of extinction:

1.4.1.1. Theories for extinction:

Two major theories that have been coined by different groups of scientists suggest various reasons for the fast-paced extinction of any taxa.

1.4.1.1.1. No genetic Factors hypothesis:

A small group of scientists believe that the extinction is the result of purely human misuse and interventions in the ecology, which has resulted in disturbances, ultimately resulting in extinction of a particular plant species. According to them, there should be little difference in genetic diversity between threatened and taxonomically related non-threatened taxa.

1.4.1.1.2. Genetic Factors Hypothesis:

Plant species, highly localized in one pocket of a given area have less genetic diversity and thereby are more likely to get swept away in conditions of environmental crisis, as compared to those, which have dispersed to various locations and thus have developed genetic variation by evolving and becoming resilient to the threats to survival. Threatened species typically have small and/or declining populations, such that inbreeding and loss of genetic diversity are unavoidable. There is extinction risk for threatened species and populations in nature (Frankham et al., 2002).

Apparent reasons for the reduction in the population size of species are habitat loss, overexploitation, impact of invasive species and pollution until they reach a point where stochastic factors further make it vulnerable to extinction (Shaffer, 1981). These Stochastic factors include demographic, environmental, and genetic stochasticity and natural catastrophes as mentioned earlier.

It has been observed that inbreeding reduces reproduction and survival rate (Frankham et al., 2002; Crnokrak & Roff, 1999) leading to reduced population heterozygosity which is associated with reduced population reproductive fitness (Reed et al., 2003), thereby increasing the risk of extinction of the species (Brook et al., 2002). Further, loss of genetic diversity reduces the ability of populations to evolve and to cope with environmental change (Frankham et al., 2002; Crnokrak & Roff, 1999; Frankham et al., 1999). Thus, reduced heterozygosity is a marker for populations with reduced reproductive fitness and an elevated risk of future extinction caused by genetic factors. Inbreeding and reduced genetic diversity were found to be associated with
higher extinction rates in populations of the plant *Clarkia pulchella* with higher versus lower inbreeding (Newman, 1997).

Conversely, if most threatened taxa do indeed show less genetic diversity than related non-threatened taxa, then this is strong evidence that genetic factors are adversely impacting these taxa (Lande, 1988; Pimm, 1991; Young, 1991; Wilson, 1992; Caro & Laurenson, 1994; Caughley, 1994; Dobson, 1999; Elgar & Clode, 2001).

The work done by Derek *et al.* in studying the impact of genetic factors on biodiversity found that most threatened taxa had lower genetic diversity than closely related non-threatened taxa, indicating reduced reproductive fitness and elevated extinction risks. These results are not compatible with the hypothesis that most species are driven to extinction before genetic factors impact them (Derek *et al.*, 2004), thus contradicting and putting aside the hypothesis of no genetic factors hypothesis.

Thus, molecular tools play an essential role in checking the luring threat on a particular plant species by studying the genetic makeup of the plant.

### 1.5. Possible solutions through modern science

The first and the foremost step that requires to be taken other than controlling unscrupulous removal of these medicinal plants from the forests is to establish systematic cultivations to ensure more supply over the growing demands by the industries.

However, relatively very few medicinal plant species are being systematically cultivated. Till the present day, the trend has been to collect it from the wild and supply it for petty money (Lange & Schippmann, 1997; Srivastava *et al.*, 1996; Xiao Pen-Gen, 1991).

There are many factors because of which this trend continues in various regions of India and the world. These factors are:

- The wild collectors do not possess much knowledge about the growth and reproduction cycle of the plants they uproot.
- Though these plants and their products are responsible for the growing spurt in pharmaceutical economics, the time, research, and experience
leading to domestication and cultivation requires funds, which are not easily made available.

- A few medicinal plants out of all enlisted have potential markets to support the inputs mentioned above.
- Systematic cultivation may provide fewer social and economic benefits than wild collection of the same as it helps securing valuable income for many rural households, especially in developing countries, and is an important factor in the source countries' local economies (Schippmann et al., 2006).

1.5.1. Plant Tissue Culture:
In such circumstances, the scientific interventions become all the more important. In the past 50 years or so, a very reliable and rapid technique to regenerate plants and to establish them both ex situ as well as in situ has been established, and which is being employed as a very efficient tool. This is Plant Tissue Culture, which has showed positive results and helped in conserving varieties of species and allowed cultivation of the same (Krogstrup et al., 1992; Wochok 1981). These in vitro culture techniques provide an alternative means of plant propagation and a tool for species improvement (Vasil, 1988).

1.5.2. Variations observed during plant tissue culture:
1.5.2.1. Genetic variations:
However, changes in ploidy level during indirect differentiation, silent mutations, changes in phenotypic or genotypic traits, etc. have been observed which are commonly named as somaclonal variations (Larkin et al., 1981). These variations are known to be heritable (Breiman et al., 1987). Other reports claim that useful morphological, cytological, and molecular variations may be generated in vitro (Larkin et al., 1989). Any system, which significantly reduces or eliminates tissue culture generated variations, can be of much practical utility. The variations may be due to several factors (Vasil 1987; 1988), such as genotypes used (Breiman et al., 1987), pathways of regeneration, and parameters employed for assessing the effect of in vitro culture, such as gross morphology and cytology (Swedlund & Vasil, 1985), field assessment, and molecular studies (Breiman et al., 1989; Chawdhury et al., 1994; Shenoy & Vasil, 1992). Thus, there is a clear need for a confirmatory technique to identify true-to-type micropropagated varieties of plants.
Micropropagation allows large-scale propagation of selected genotypes. However, problems with somaclonal variation have been encountered. Somaclonal variation refers to any phenotypic or genotypic modifications that arise from *in vitro* culture (Larkin & Scowcroft, 1981). Somaclonal variation is associated with several types of genomic disorder, including changes in ploidy (Breimann *et al.*, 1989), DNA methylation modifications, point mutations (Larkin *et al.*, 1989), insertion of mobile DNA elements or retro elements (McKenzie *et al.*, 2002) and chromosomal rearrangements (Phillips *et al.*, 1994, Isabel *et al.*, 1996). Although a few somaclonal variations are stable and genetically inherited, others are not inherited or are reversible and inherited in a non-Mendelian fashion.

The frequency of somaclonal variation in tissue culture-derived plants depends on the type of explant (Karp *et al.*, 1984), the genotype and species (Wilhelm *et al.*, 2005). In addition, the type of regeneration process (e.g., somatic embryogenesis or organogenesis) may also be a contributory factor determining the frequency of somaclonal variations (Armstrong & Phillips, 1988). *In vitro* induced stress during cellular reprogramming by Plant Growth Regulators such as 2,4-di chloro phenoxy acetic acid (2,4-D) can cause somaclonal variation. Some authors have reported that prolonged time in *in vitro* culture can promote somaclonal variation (Orton 1985; Lee & Phillips, 1987; Hartmann *et al.*. 1989; Tremblay *et al.*, 1999; Etienne & Bertrand, 2003).

A number of PCR-based techniques for analysing somaclonal variations have been tested including AFLP (Amplified Fragment Length Polymorphism), RAPD (Random Amplification of Polymorphic DNA) and SSR (Simple Sequence Repeats). However, when evaluating somaclonal variation, it is usually more advantageous to use more than one DNA amplification technique (Palombi & Damiano, 2002).

The AFLP technology originally proposed by Vos *et al.* (1995) detects polymorphisms in different genomic regions, between 50 and 100 fragments at the same time. It is a highly sensitive and reproducible technique and no prior sequence information for amplification is needed. As a result, AFLP has become widely used for studying genetic variation in strains or closely related species of bacteria, fungi, plants and animals (Savelkoul *et al.*, 1999).
1.5.2.1.1. Need of molecular investigations in medicinal plants:
One very well known fact is that with change in geographic and climatic conditions the capacity of a medicinal plant species to express its active principal compound varies (Oleszek et al., 2002). Many researchers have studied geographical variation at the genetic level. This is also significant in designing conservation strategies for plants. RAPD-based molecular markers have been found to be useful in differentiating different accessions of *Taxus wallichiana*, *Azadirachta indica*, *Juniperus communis* L., *Codonopsis ilosula*, *Allium schoenoprasum* L., *Andrographis paniculata* collected from different geographical regions (Shasany et al., 1999; Farooqui et al., 1998; Adams et al., 2002; Fu et al., 1999; Fadmesh et al., 1999). ISSR markers have been used for different accessions of *Cannabis sativa* and *Arabidopsis thaliana* L. Heynh (Kojoma et al., 2002; Barth et al., 2002).

RAPD and RFLP have been used for interspecies variations also for different genera such as *Glycerrhiza*, *Echinacea*, *Curcuma* and *Arabidopsis* (Yamazaki et al., 1994; Kapteyn et al., 2002; Chen et al., 1999; Lind-Hallden et al., 2002). RAPD and RFLP have also been applied for characterization of *Epimedium*, *Scutellaria*, three subspecies of *Melissa officinalis*, *Hibiscus cannabinus* L., *Capsicum annum*, *Simmondsia chinensis* L. Schneider, *Vitis vinifera* L. and tea (*Camellia sinensis*) (Nakai et al., 1996; Hosokawa et al., 2000; Wolf et al., 1999; Cheng et al., 2002; Prince et al., 1995; Amarger and Mercier 1995; Tessier et al., 1999; Wachira et al., 1995).

Genetic diversity and phylogenetic studies have been undertaken for plants like *Podophyllum peltatum*, *Withania* species, Papaya and its wild relatives of the family Caricaceae, *Brassica campestris* cultivars, *Eucalyptus grandis* and *Eucalyptus urophyll*, *Taxus bravifolia* Nutt. using AFLP and RAPD markers (Lata et al., 2002; Negi et al., 2000; Van et al., 2002; Das et al., 1999; Grattapaglia and Sederoff, 1994; Gocmen et al., 1996). Phylogenetic relationship has been studied among citrus and its relatives using SSR markers (Pang et al., 2003).

Recently these molecular tools have been employed for medicinal plant cultivation and breeding too. ISSR–PCR has been found to be an efficient and reliable technique for the identification of zygotic plantlets in citrus interploid crosses, *Hypericum perforatum*, a well known medicinal plant (Tusa et al., 2002; Steck et al., 2001).
1.5.2.2. Other molecular variations:
These genetic variations ultimately may or may not result in production of a novel/ different protein in the system. Several proteins are expressed and silenced during the process of differentiation at different stages. During *in vitro* growth, with the onset of morphogenesis, certain proteins are expressed as and when the plant organ requires them in the amount required. However, changes in ploidy level may significantly alter the protein profile of a tissue.

1.5.2.3. Phytochemical variations:
Pharmacognosy, which was once known as a field of chemical applications of plants, with recent advances and demand of drug designing and discovery techniques, have also become multidisciplinary resorting to these molecular markers for their characterizatation. Chinese researchers have applied DNA markers extensively for characterization of botanicals from the Chinese Materia Medica for quality control measures. However, while the DNA fingerprint of the plant remains same irrespective of the plant part used, its phytochemical fingerprinting would change with different parts or with change in variation in climatic conditions. Thus, molecular tools can ensures presence of the correct genotype but does not ensure presence of the same chemical constituents. Several attempts have been made in recent years, to correlate DNA markers with qualitative and quantitative variations in phytochemical composition among closely related species. However, proper integration of both these techniques is required to establish quality controls for plant species.

1.5.3. Molecular Tools to verify genetic make up:
Development of these molecular tools, especially molecular markers have contributed greatly to our knowledge of plant genetics and have revolutionized our understanding in developing linkage maps between species or mapping a particular species. These maps have been of great utility in identifying markers linked to genes of economical, medicinal or horticultural and agronomical importance. Insights about the genome of a particular species, evolutionary changes and interspecies relationship, conservation and population strategy designing, etc. are among other advantages of these tools.

Types of DNA markers used in plant genome analysis: Various types of DNA-based molecular techniques (Joshi *et al.*, 1999; Powell *et al.*, 1996; Botstein *et al.*, 1980) are utilized to evaluate DNA polymorphism. These are hybridization-based methods, Polymerase Chain Reaction (PCR)-based methods and sequencing-based methods.
1. Hybridization-based Methods: Hybridization-based methods include restriction fragment length polymorphism (RFLP) (Botstein et al., 1980) and variable number tandem repeats (VNTR) (Nakamura et al., 1987). Labelled probes such as random genomic clones, cDNA clones, probes for microsatellite (Litt & Luty, 1989) and minisatellite (Jeffrey et al., 1985) sequences are hybridized onto the filters that contain restriction enzyme digested DNA. Presence and absence of bands upon hybridization can help in finding out the rate of polymorphism.

2. PCR-based Methods: Polymerase Chain Reaction is mainly used for amplification of DNA. PCR-based markers involve in vitro amplification of particular DNA sequences or loci, with the help of specific or arbitrary or random oligonucleotide primers and the thermostable DNA polymerase enzyme. PCR-based techniques where random primers are used are: Random Amplified Polymorphic DNA (RAPD), (Williams et al., 1990; Welsh & McClelland, 1990) arbitrarily primed PCR (AP-PCR) that uses arbitrary primers (Welsh & McClelland, 1991) and DNA amplification fingerprinting (DAF) (Caetano-Anolles et al., 1991; Caetano-Anolles & Bassam 1993). Inter simple sequence repeats (ISSRs) (Zietkiewicz et al., 1994) polymorphism is a specific primer-based polymorphism detection system, where a terminally anchored primer specific to a particular simple sequence repeat (SSR) is used to amplify the DNA between two opposed SSRs of the same type.

Polymorphism occurs whenever one genome is missing in one of the SSRs or has a deletion or insertion that modifies the distance between the repeats. A recent approach known as amplified fragment length polymorphism (AFLP) (Zabeau, 1993; Vos et al., 1995) is a technique that is based on the detection of genomic restriction fragments by PCR amplification. Adaptors are ligated to the ends of restriction fragments followed by amplification with adaptor-homologous primers. AFLP has the capacity to detect thousands of independent loci and can be used for DNAs of any origin or complexity (Kumar 1999).

The three most common types of markers used today are RFLP, RAPD and isozymes. Of the three marker types, RFLPs have been used the most extensively. However, RFLP markers have several advantages in comparison with the RAPD and isozyme markers, the latter two techniques are also equally used to check heterozygosity and gene sequence. A brief description of each of these techniques has been given below.
RFLP (Restriction Fragment Length Polymorphism): It is a molecular marker based on the differential hybridization of cloned DNA to DNA fragments in a sample of restriction enzyme digested DNAs; the marker is specific to a single clone/restriction enzyme combination.

RAPD (Randomly Amplified Polymorphic DNA): It is a molecular marker based on the differential PCR amplification of a sample of DNAs from short oligonucleotide sequences.

AFLP (Amplified Fragment Length Polymorphism): It is a molecular marker generated by a combination of restriction digestion and PCR amplification.

SSR (Simple Sequence Repeat): It is a molecular marker sequence consisting largely of a tandem repeat of a specific k-mer (such as (CA) 15), which are in many cases polymorphic and have been widely used in genetic mapping.

ISSR (Inter-simple sequence repeat): ISSR primers anchored at their 3' ends direct the amplification of the genomic segments between the ISSRs.

Table 1.3 enumerates advantages of some of these techniques.

**Table 1.3. Comparison of some of the PCR based amplification techniques:**

<table>
<thead>
<tr>
<th>Feature</th>
<th>RFLP</th>
<th>RAPD</th>
<th>AFLP</th>
<th>SSR</th>
<th>Sequencing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Development cost</td>
<td>Medium</td>
<td>Low</td>
<td>Low</td>
<td>High</td>
<td>High</td>
</tr>
<tr>
<td>Running Cost</td>
<td>High</td>
<td>Low</td>
<td>Medium</td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>Samples/day</td>
<td>Low</td>
<td>High</td>
<td>High</td>
<td>High</td>
<td>Low</td>
</tr>
<tr>
<td>Skill required</td>
<td>Low</td>
<td>Low</td>
<td>Medium</td>
<td>Low medium</td>
<td>High</td>
</tr>
<tr>
<td>Automation</td>
<td>Difficult</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Radioactivity</td>
<td>Yes-No</td>
<td>No</td>
<td>Yes-No</td>
<td>Yes-No</td>
<td>Yes-No</td>
</tr>
<tr>
<td>Reliability</td>
<td>High</td>
<td>Low-medium</td>
<td>High</td>
<td>High</td>
<td>High</td>
</tr>
<tr>
<td>Dominant/Codominant</td>
<td>Codominant</td>
<td>Dominant</td>
<td>Dominant</td>
<td>Codominant</td>
<td>Codominant</td>
</tr>
<tr>
<td>Polymorphism</td>
<td>Medium</td>
<td>Medium</td>
<td>Medium</td>
<td>High</td>
<td>Medium-low</td>
</tr>
</tbody>
</table>

3. Isozymes: This is a molecular marker system based on the staining of proteins with identical function, but different electrophoretic mobilities. They are basically protein markers.

Sequencing-Based Markers:
Sequencing of DNA of a particular species can serve as an identification method too. As mentioned earlier, polymorphisms and variations in genetic sequence can occur due to transversion, insertion or deletion. These variations can be traced directly and information on a defined locus can be obtained. Genetic variation occurs extensively at the single nucleotide level. Direct sequencing can efficiently identify such single nucleotide polymorphisms that usually depend on the closeness of the species under investigation. Other sequencing-based strategies include analysis of the variable internal transcribed spacer (ITS) sequences of ribosomal DNA (rDNA). The ITS region of 18s-26s rDNA has proved to be a useful sequence for phylogenetic studies in many angiosperm families. The level of ITS sequence variation suitable for phylogenetic analysis is found at various taxonomic levels within families, depending on the linkage.

A number of researchers have also sequenced other regions of DNA such as trnK of chloroplast and spacer region of 5s rDNA as diagnostic tools for authentication purpose (Yang et al., 2001).

Thus, medicinal plants have become a core subject of study having various applications of the same in different fields. We have undertaken an exploratory study on the use of molecular markers for quick identification of botanical materials by establishing some preliminary DNA fingerprints and by comparing the genotypic pattern of micropropagated plants with their natural mother plants. Our strategy involves identification of species-specific marker after screening a number of species and/or varieties of the medicinal plant using random oligonucleotide primers, followed by cloning and subsequently converting it to markers for better specificity and reproducibility.

1.6. The need of the hour: DNA barcoding of plants containing active medicinal compounds:
DNA barcoding is a tool for rapid species identification based on DNA sequences it shares an emphasis on large scale genetic data acquisition with genomics that offers new answers to questions previously beyond the reach of traditional disciplines. DNA barcodes consist of a standardized short sequence of DNA (400–800 bp) that in principle should be easily generated and characterized for all species on the planet. A massive on-line digital library of barcodes are being
created which will serve as standards to which the DNA barcode sequence of an unidentified sample from the forest, garden, or market can be matched. Similar to genomics, which has accelerated the process of recognizing novel genes and comparing gene function, DNA barcoding will allow users to efficiently recognize known species and speed the discovery of species yet to be found in nature. DNA barcoding aims to use the information of one or a few gene regions to identify all species of life, whereas genomics, the inverse of barcoding, describes in one (e.g., humans) or a few selected species the function and interactions across all genes.